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703.CELLULAR IMMUNOTHERAPIES: BASIC AND TRANSLATIONAL

Gamma-Delta ($\gamma\delta$) CAR-T Cells Lacking the CD3z Signaling Domain Enhance Targeted Killing of Tumor Cells and Preserve Healthy Tissues

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Introduction: Chimeric antigen receptor T cell (CAR-T) therapy has shown remarkable efficacy against B cell malignancies for patients who have limited treatment options. Successful advancement of CAR-T therapy to myeloid hematopoietic and solid malignancies, however, has been limited by the potential of life-threatening on-target, off-tumor toxicities against healthy tissues, the natural tumor heterogeneity and consequent selection pressure leading to relapse. Addressing these limitations to widen the therapeutic index is crucial as we seek safer and more effective CAR-T therapies. In this regard, $\gamma\delta$ T cells as first responders of immunity hold promise as they can directly identify and eliminate malignant cells through the recognition of multiple tumor-associated stress antigens not commonly expressed on normal tissues. We sought to combine the tumor-sensing capabilities of $\gamma\delta$ T cells and the localization enhancement of CAR-T by excluding the CD3ζ domain in a non-signaling CAR (nsCAR) to allow the targeted killing against tumors, while sparing healthy tissues.

Methods: Standard and nsCAR constructs against CD19, CD33 and CD123 were cloned into separate second-generation lentiviral vectors. The CAR constructs contained either a scFv targeting the respective antigen (anti-CD19/anti-CD33) or an IL-3 zetakine (IL-3z) targeting the CD123 followed by a flag-tag, a hinge, a transmembrane domain and a costimulatory domain + CD3ζ. IL-15 co-expression was incorporated to enhance $\gamma\delta$ T cell fitness and persistence. CAR activation was determined by Jurkat T cells co-cultured with target ALL or AML cell lines followed by lentivector transduction of activated and expanded Vδ2$^+$ $\gamma\delta$ T cells. Cellular cytotoxicity was assessed at multiple E:T ratios against respective leukemia cell lines and healthy peripheral blood cells expressing the target antigen.

Results: We observed significant upregulation of CD69 surface expression indicating activation of CD3ζ+ CD19CAR/CD33CAR/IL-3z Jurkat cells (3x, 20x, and 2x respectively) following culture with Nalm6 (CD19) and KG-1 (CD33 & IL-3z). CD3ζ- ns19CAR/ns33CAR/nsIL-3z Jurkat cells did not upregulate CD69 in parallel cultures. We also observed a time-dependent reduction of the CD3ζ+ CD33 CAR$^+$ population over 7 days (43%) in Jurkat cells following extended coculture with KG-1 cells, while the nsCAR$^+$ Jurkat population remained unchanged, indicating the nsCAR may mitigate activation induced cell death (AICD).

Ex vivo activated $\gamma\delta$ T cells from healthy donors (N=4), were transduced with nsCAR lentiviral vectors with high efficiency (up to 80%). The ns19CAR cells effectively killed CD19$^+$ Nalm6 cells and demonstrated enhanced (>1.5x) cytotoxicity compared to untransduced $\gamma\delta$ T cells (UTD). After 48hr co-culture (E:T=2:1), the ns19CAR cells killed 79.7%+6.6% Nalm6 cells compared to 46.1%+7.2%) with UTD. Minimal cytotoxicity was observed for ns19CAR and UTD $\gamma\delta$ T cells against B cells from healthy donor PBMC 5.2%+6.6% for ns19CAR vs. to -4.6%+7.7% with UTD. No significant difference in cytotoxicity against the CD19- K562 cells between the UTD or ns19CAR cells (73.5% vs. 71.5%, E:T=2:1) suggesting the enhanced cytotoxicity is ns19CAR directed. Similarly, ns33CAR and nsIL-3z cells, although requiring higher E:T ratios for similar killing efficiency, continued to demonstrate enhanced (up to 2.0x) killing against AML cells (HL-60, KG-1 and MOLM13) and CML cells K-562 compared to UTD in cytotoxicity assay with 24hr co-culture. Meantime, minimal cytotoxicity (<10%) was observed for ns33CAR, nsIL-3z, or UTD $\gamma\delta$ T cells against CD33+ cells isolated from healthy donor PBMC.

Conclusions: Ex vivo activated nsCAR cells efficiently recognize and kill leukemia cell lines while sparing peripheral blood cells bearing the same target antigen. The nsCAR cells also show increased cytotoxicity against leukemias over unmodified activated $\gamma\delta$ T cells suggesting improvement in tropism and/or binding efficiency. In summary, our findings showed that the combination of nsCAR on $\gamma\delta$ T cells may increase the therapeutic index to allow expansion of CAR-T therapy to cancers with unacceptable target expression on critical healthy cell populations. Further optimization of the nsCAR constructs may potentially enhance both the efficacy and safety profile of the next generation adoptive cell therapies against wider selection of cancers.

Disclosures Ding: *IN8bio:* Current Employment. **Li:** *IN8bio:* Current Employment. **Li:** *IN8bio:* Current Employment. **ter Haak:** *IN8bio:* Current Employment. **Lamb:** *IN8bio:* Current Employment, Current equity holder in publicly-traded company.

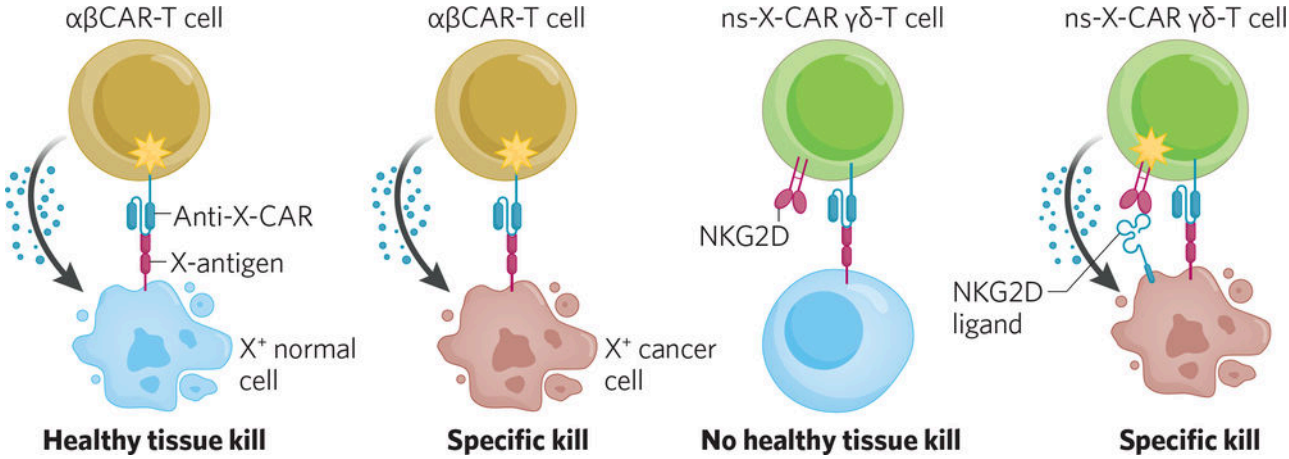

Figure 1

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